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LIM-kinase is critical for the mesenchymal-to-amoeboid cell morphological transition in 3D matrices

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ABSTRACT

Tumor cells can migrate in 3D matrices in either a mesenchymal-like or amoeboid mode. HT1080 fibrosarcoma cells cultured in 3D collagen gels change their morphology from mesenchymal-like (elongated) to amoeboid (round) following protease inhibitor (PI) treatment or active Rho or ROCK expression. In this study, we examined the role of LIM-kinase 1 (LIMK1) in the PI- or Rho/ROCK-induced cell morphological change. We showed that LIMK1 was activated after PI treatment of HT1080 cells in 3D collagen gels and this activation was blocked by a ROCK inhibitor. While overexpression of LIMK1 induced cell rounding, knockdown of LIMK1 or the expression of kinase-inactive LIMK1 suppressed PI- or Rho/ROCK-induced cell rounding. These results suggest that LIMK1 plays an essential role in the PI- or Rho/ROCK-induced mesenchymal-to-amoeboid cell morphological transition of HT1080 cells cultured in 3D collagen gels. Furthermore, LIMK1 knockdown suppressed the invasive activity of HT1080 cells in collagen gels with or without PIs, indicating that LIMK1 mediates both the mesenchymal and amoeboid modes of invasion of HT1080 cells.

Introduction

Malignant tumor cells *in vivo* migrate through the 3D extracellular matrix (ECM) barrier to invade and metastasize. Recent studies indicate that tumor cells can migrate in 3D matrices in at least two distinct migration modes, mesenchymal-like and amoeboid, depending on the cell type, state, and environmental conditions [1–3]. Cells migrating in a mesenchymal-like mode have an elongated shape with lamellipodium-like protrusions. These cells move by a path-generating mechanism involving ECM proteolytic degradation at the leading edge by the actions of extracellular proteases, such as matrix metalloproteases (MMPs) [3]. In contrast, cells migrating in an amoeboid mode have a round shape, and move by a path-finding mechanism involving the protease-independent squeezing of cells through ECM gaps by the generation of bleb-like membrane protrusions and actomyosin-based contractile forces [3]. Some tumor cells can switch between the two migration modes when moving in 3D matrices [1–6].

HT1080 cells predominantly have an elongated morphology and exhibit mesenchymal-like migration in 3D gels, but they switch to a round morphology and amoeboid migration mode after protease inhibitor (PI) treatment [1]. The ability of tumor cells to switch from a mesenchymal (protease-dependent) to an amoeboid (protease-independent) migration mode may partly account for the limited therapeutic effect of MMP inhibitors against tumor cell dissemination [7]. Recent studies indicate that RhoA and its downstream kinase ROCK play critical roles in the mesenchymal-amoeboid transition and the amoeboid motility of tumor cells by promoting myosin light chain (MLC) phosphorylation, which in turn generates the actomyosin-based contractile force required for amoeboid movement [2–6].

Actin cytoskeletal remodeling is essential for cell migration and morphological changes. Cofilin is a key regulator for controlling actin filament dynamics and reorganization by stimulating the depolymerization and severing of actin filaments [8]. Cofilin is inactivated by Ser-3 phosphorylation by LIM-kinases (LIMKs) [9,10], and is reactivated by dephosphorylation by Slingshot (SSH) family protein phosphatases [11]. Several lines of evidence suggest that LIMK1 plays a critical role in cell migration and tumor cell invasion [12–16]. Since LIMK1 is activated by ROCK-catalyzed phosphorylation [17,18], LIMK1 may mediate the elongated-to-round cell morphological change and mesenchymal-amoeboid transition of migration mode transition of tumor cells in 3D matrices. However, little is known about the role of LIMK1 in cell morphology and migration in 3D environments.

In this study, we examined the role of LIMK1 in the PI-induced elongated-to-round cell morphological transition of HT1080 cells

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; LIMK, LIM-kinase; MLC, myosin light chain; MMP, matrix metalloprotease; PI, protease inhibitor; SSH, Slingshot; WT, wild-type.

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in 3D collagen matrices. We show that LIMK1-mediated cofilin phosphorylation plays an essential role in PI-induced and RhoA/ROCK-mediated cell morphological changes. We also provide evidence that LIMK1 is crucial for both the mesenchymal and amoeboid cell migration modes in 3D collagen gels.

Materials and methods

Materials. Y27632 (ROCK inhibitor) and GM6001 (MMP inhibitor) were purchased from Calbiochem. E64 (Cys protease inhibitor), aprotinin (Ser protease inhibitor), and puromycin were from Sigma. Luepeptin (Ser and Cys protease inhibitor) was from Peptide Institute (Minoh, Japan). The mixture of PIs used contained 20 μM GM6001, 250 μM E64, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. An anti-LIMK1 antibody was prepared as described [19].

Plasmid construction. Expression plasmids encoding cyan fluorescent protein (CFP)-tagged human LIMK1 or its kinase-dead D460A mutant, as well as mDsRed-tagged cofilin and its mutants, were constructed as described previously [16,20]. Plasmids for CFP-RhoA(G14 V) and CFP-ROCKΔ3 were constructed by inserting their cDNAs into the pECFP-C1 vector. The shRNA-targeting constructs were generated using pSUPER or pSUPER.retro.puro vector plasmids (Oligoengine, Seattle, WA), as described previously [16]. The 19-base sequences targeting human LIMK1 was 5′-GAATGTGG TGGTGGCTGAC-3′. A control shRNA plasmid was constructed by substituting two bases in the target sequence (5′-GAATGTTGTGGT GGCTGCC-3′).

Cell culture, transfection, and retrovirus infection. HT1080 human fibrosarcoma cells (ATCC; CCL-121) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were transfected with expression plasmids or shRNA plasmids using FuGENE6 (Roche Applied Science). To generate retroviral supernatants, GP2-293 packaging cells were transfected with pCMV-VSV-G and pSUPER.retro.puro plasmids using FuGENE6. The culture medium was centrifuged 48 h after transfection, and the viral supernatant was used for infection after adding 8 μg/ml polybrene. Infected HT1080 cells were cultured for 24 h, washed, and selected by culturing with 1 μg/ml puromycin for about 1 week.

Cell morphology assay. For 3D culture, HT1080 cells were suspended in solution of Type I collagen (Cellmatrix I-C, Nitta gelatin, Osaka, Japan), prepared at a final concentration of 1.7 mg/ml, according to the manufacturer's protocol. Cells were incorporated in a collagen gel and stratified on a collagen gel-precoated glass bottom dish, and then cultured for 12 h. Cells were treated with or without the PI mix for 24 h and fixed with 4% paraformaldehyde. Fluorescence images were obtained using a confocal microscope (TSP SP2, Leica). The optical sections captured every 2 μm based on YFP fluorescence were reconstructed to generate the 3D image of cells. A cell was counted as round when the length of its longest axis was less than twice that of its shortest axis.

In vitro kinase assay. LIMK1 was immunoprecipitated with an anti-LIMK1 antibody and subjected to an *in vitro* kinase reaction using $(His)_6$-cofilin as a substrate, as described previously [18]. The reaction mixtures were separated by SDS–PAGE and analyzed with autoradiography to measure ^{32}P-labeled $(His)_6$-cofilin.

Invasion assay. For the cell invasion assay, the lower wells of a Transwell culture chamber (8-μm pore size; Costar #3422) were filled with DMEM containing 10% fetal calf serum. HT1080 cells (1×10^5 cells) suspended in 100 μl of serum-free collagen solution were loaded into the upper wells. After gelation, the upper chamber was filled with serum-free DMEM. The PI mix was added to the collagen gel and DMEM and added again to DMEM 24 h after incubation. After incubation for 48 h, the cells were fixed and stained with DAPI. The cells that had migrated into the lower wells were counted and are reported as the percentage of the input cells.

Results

LIMK1 is activated by PI treatment of HT1080 cells in 3D matrices

HT1080 fibrosarcoma cells cultured in 3D collagen gels predominantly exhibited a mesenchymal-like (elongated) morphology in the absence of PIs, but most cells transitioned to the amoeboid (round) type after PI treatment (Fig. 1A), as reported previously [1]. To examine the role of LIMK1 in the PI-induced cell morphology change, we first analyzed the kinase activity of LIMK1 in PI-treated and untreated HT1080 cells cultured in 3D gels. *In vitro* kinase assays revealed that the LIMK1 kinase activity was increased 1.7-fold in PI-treated cells, compared to untreated cells (Fig. 1B). Treatment with Y27632, a specific inhibitor of ROCK, completely blocked PI-induced LIMK1 activation (Fig. 1B). These results indicate that LIMK1 is activated in a ROCK-mediated manner by the PI treatment of HT1080 cells in 3D matrices.

LIMK1 overexpression induces cell rounding

To investigate whether LIMK1 is involved in cell rounding in 3D matrices, we next examined the HT1080 cell morphological effects of overexpressing LIMK1 or its kinase-inactive D460A mutant. HT1080 cells transfected with CFP or CFP-tagged LIMK1 (WT or D460A) were cultured in 3D collagen gels and then treated with the PI mix or left untreated. After fixing, transfected cells were visualized by fluorescence and the percentages of round cells among the CFP-positive cells were determined. For cells expressing control CFP, 27% of cells were round in the absence of PIs. This percentage increased to 57% after PI treatment, indicating that the PI mix promoted cell rounding (Fig. 2A). When HT1080 cells were transfected with LIMK1(WT)-CFP, 58% of cells exhibited a round cell morphology in the absence of PIs, and this percentage slightly increased after PI treatment (Fig. 2A). In contrast, LIMK1(D460A)-

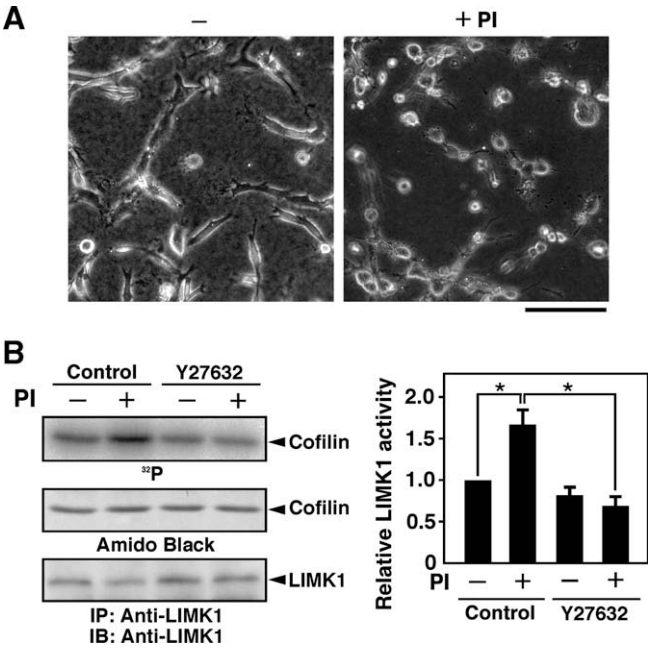

Fig. 1. LIMK1 is activated by PI treatment. (A) PI-induced cell shape change. HT1080 cells were cultured for 12 h in 3D collagen gels with or without PIs, fixed, and imaged by phase-contrast microscopy. Scale bar, 100 μm. (B) PI-induced LIMK1 activation. HT1080 cells were cultured for 12 h in 3D collagen gels with or without PIs and 10 μM Y27632. Cells in 3D gels were lysed and LIMK1 was immunoprecipitated (IP) and subjected to an *in vitro* kinase assay, as described [18]. The relative kinase activities of LIMK1 are shown as means ± S.D. of three independent experiments. $^*p < 0.05$.